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Effects of early developmental conditions on innate immunity are only evident under favourable adult conditions in zebra finches

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Abstract

Long-term effects of unfavourable conditions during development can be expected to depend on the quality of the environment experienced by the same individuals during adulthood. Yet, in the majority of studies, long-term effects of early developmental conditions have been assessed under favourable adult conditions only. The immune system might be particularly vulnerable to early environmental conditions as its development, maintenance and use are thought to be energetically costly. Here, we studied interactive effects of favourable and unfavourable conditions during nestling and adult stages on innate immunity (lysis and agglutination scores) of captive male and female zebra finches (*Taeniopygia guttata*). Nestling environmental conditions were manipulated by a brood size experiment, while a foraging cost treatment was imposed on the same individuals during adulthood. This combined treatment showed that innate immunity of adult zebra finches is affected by their early developmental conditions, and varies between both sexes. Lysis scores, but not agglutination scores, were higher in individuals raised in small broods and in males. However, these effects were only present in birds that experienced low foraging costs. This study shows that the quality of the adult environment may shape the long-term consequences of early developmental conditions on innate immunity, as long-term effects of nestling environment were only evident under favourable adult conditions.

Keywords brood size manipulation, complement, developmental stress, foraging costs manipulation, immunocompetence, natural antibodies

Introduction

Adverse conditions experienced during early life, such as suboptimal nest temperature, nutritional stress and exposure to pathogens and toxins, have been shown to negatively affect an individual's physiology, survival and reproductive success during adulthood in a wide range of species (e.g. Gluckman and Hanson 2004; Lindström 1999; Lummaa and Clutton-Brock 2002; Metcalfe and Monaghan 2001). Long-term consequences of early developmental conditions have, however, been found to vary with the quality of the adult environment in opposing ways (reviewed in Monaghan 2008). First, it has been demonstrated that nestlings raised under poor conditions suffer a suboptimal development and experience negative effects irrespective of the quality of the adult environment (Grafen 1988; Monaghan 2008). Second, several studies found that long-term effects of developmental stress remain masked under favourable adult conditions as physiological responses are not affected by an individual's quality (e.g. Hendrickx et al. 2003; Lens et al. 2002; Talloen et al. 2010). However, when environmental conditions deteriorate and individuals become challenged, qualitative differences become reflected in their physiological responses, ultimately leading to variation in survival (e.g. Hendrickx et al. 2003; Lens et al. 2002; Talloen et al. 2010). Third, individuals developing under unfavourable circumstances may cope better with poor conditions during adulthood because of the similarity between early and later environments. If this is the case, a reverse pattern can be expected when adult conditions are more favourable, as organisms raised in poor environments are not developmentally adapted to more favourable ambient conditions (Monaghan 2008).

As the development, maintenance and use of an organism's immune system is assumed to be energetically costly (e.g. Klasing 2004; Lochmiller and Deerenberg 2000; Norris and Evans

2000; Sheldon and Verhulst 1996), immune function can be expected to be suppressed in large broods because of higher nutritional stress and increased sibling competition (e.g. Naguib et al. 2004; Saino et al. 1997). Yet, poor immunity can come at a price as the immune system is the main physiological mechanism to resist disease, and hence, to safeguard future survival and reproduction (reviewed in Møller and Saino 2004). This might be particularly true for two components of the constitutive innate immune system, the natural antibodies (hereafter NAbs) and complement cascade, which provide the first defence mechanisms against a broad spectrum of pathogens, and therefore seem to play a crucial role in enhancing an organism's survival (Ochsenbein and Zinkernagel 2000). NAbs (mostly IgM) are already present in individuals that have not been exposed to antigens (Bandeira et al. 1988). Their function is to bind to a particular antigen or pathogen, to clear foreign substances and to initiate the complement system, which consists of circulating serum proteins that are sequentially activated to result in cell lysis (Boes 2000 and references therein). Although NAbs and the complement cascade are assumed to interact, their concentrations are usually not, or only weakly, correlated (De Coster et al. 2010; Mendes et al. 2006; Møller and Haussy 2007; Soler et al. 2007, but see Parejo et al., 2007). Despite the theoretical expectations outlined above, empirical evidence of effects of early developmental conditions on innate immune function remains scarce and heterogeneous. For example, while complement activation was positively related to clutch size in a suite of tropical bird species, no such effect was found for NAbs (Lee et al. 2008). In a study on European rollers (*Coracias garrulus*), both components varied with brood hierarchy in nestling body mass, though in opposite ways. While lighter nestlings had more NAbs compared to heavier ones, the former showed less complement activity (Parejo et al. 2007). Given the role of constitutive innate immunity as a first line defence against infections and its effects on survival (Ochsenbein and Zinkernagel 2000), it is important to gain more general insights into the long-term effects of

developmental conditions, in addition to their interaction with the adult environment, on this branch of the immune system. Furthermore, it remains unexplored whether such effects on innate immunity differ between the sexes, despite indications that inflammatory immune responses in reaction to conditions during development and adulthood differ between male and female mallard ducks (*Anas platyrhynchos*; Butler and McGraw 2011).

Here we attempt to explore this research question by experimentally manipulating environmental conditions during the nestling and adult stages in captive male and female zebra finches (*Taeniopygia guttata* Vieillot) and study effects on their innate immune capacity. Brood size manipulations are a well-known technique to manipulate nutritional intake and the level of competition of individual nestlings (Griffith and Buchanan 2010). At a short term, being raised in a large brood reduces body sizes, growth rates, phytohaemagglutinin (PHA) inflammatory responses and survival of nestlings (e.g. Alonso-Alvarez et al. 2006; de Kogel 1997b; Naguib et al. 2004; Tschirren et al. 2009), while at a longer term, it affects adult body size, metabolic rates, inflammatory responses and reproductive success (e.g. Alonso-Alvarez et al. 2006; Tschirren et al. 2009; Verhulst et al. 2006). Early developmental conditions can further be expected to differentially affect adult innate immunity in male and female zebra finches, as the latter have been shown to be more negatively affected by harsh rearing conditions (e.g. Bradbury and Blakey 1998; de Kogel 1997b; Kilner 1998; Martins 2004; Verhulst et al. 2006), and inflammatory and humoral immune responses have been shown to vary between sexes and contexts (Love et al. 2008; McGraw and Ardia 2005). Furthermore, increased foraging costs in adulthood result in physiological adjustments to save energy (Wiersma et al. 2005; Wiersma and Verhulst 2005), and might therefore also affect innate immunity because of the associated energetic costs (Klasing 2004).

The majority of studies on long-term fitness effects of development under poor conditions were conducted under favourable adult conditions (reviewed in Monaghan 2008), which precludes the evaluation of possible interactions between early and later life conditions. Here we consider favourable and unfavourable adult conditions and study the interactive effects with manipulations of early condition. Nestling environmental conditions were manipulated through a brood size experiment, while the same individuals were subsequently subjected to a foraging cost treatment during adulthood. Based on these paired experiments, we examined if, and to what extent: (i) environmental conditions during the nestling and/or adult stage have persistent effects on adult innate immune responses; (ii) long-term effects of nestling environment depend on the quality of the adult environment; and (iii) effects of environmental conditions differ between males and females.

Material and methods

BIRD HUSBANDRY AND EXPERIMENTAL DESIGN

Our study was conducted between 2006 and 2009 in a captive population kept at the University of Groningen, The Netherlands. All individuals were hatched under standard laboratory conditions and subjected to a cross-fostering at the age of 5 days such that nestlings were either raised in a small brood (2 nestlings) or a large brood (6 nestlings). In this procedure we ensured that parents rearing small or large broods did not on average differ in reproductive traits such as clutch size or brood size before manipulation. We can therefore safely assume that parents rearing small or large broods did not on average differ in their ability to care for a brood. Birds from which the brood size had not been manipulated (i.e. comprising two or six nestlings in the original brood) were excluded from our analyses. Experimental brood sizes fell within the range normally observed in wild (Zann 1996) and laboratory (Naguib et al. 2004) populations. We manipulated the brood size towards the extremes of the normal range in order to trigger sufficient variation in nestling environment between the two experimental groups. When individuals were between 3-23 months old, they were distributed over eight single-sex outdoor aviaries (four with males only, four with females only). Birds were kept in same sex groups because the opportunity to invest in sexual interactions could mitigate treatment effects on physiology and life span when birds in the poor foraging environment (see further) engage less in such interactions. Individuals were divided equally over the aviaries with respect to the experimental brood sizes and their age. In this way, each aviary contained 32-37 individuals of different age and brood size treatments and was exposed to a natural light-dark cycle and ambient temperature. While drinking water and tropical food seed mixture was supplied *ad libitum* in each aviary, foraging costs per reward were manipulated by placing perches under the food container in four cages (two per

sex; *low foraging cost* treatment) while not placing them in four other cages (two per sex; *high foraging cost* treatment). This treatment forced birds to fly back and forth between a perch and the food container for each seed they consumed. Seeds that fell from the seed containers were not accessible to prevent individuals from each aviary to forage on spilled seed at low cost. Previous work on the same birds showed that birds experiencing high foraging costs spent more than twice as much time foraging and significantly reduced basal metabolic rates compared to low foraging cost birds, demonstrating the effectiveness of the imposed treatment to increase foraging costs and to affect physiological responses (Koetsier and Verhulst 2011). Upon blood collection, survival rates were lower in high-cost foragers raised in large broods (39 out of 69 individuals, 57%) compared to those raised in small broods (53 out of 64, 83%) (P=0.003). Rates did not differ among low-cost foragers (small: 42 out of 58, 72%, large: 50 out of 75, 67%; P=0.50), while the interaction between brood size and foraging costs was marginally significant (P=0.077; generalized linear mixed model; see below for random effects). A total of 96 females and 88 males that were still alive in October 2009 (16-45 months old and 8-22 months subjected to the foraging cost treatment) were weighed, and 100-150 µl blood was collected in heparinized capillary tubes via brachial vein puncture with a sterile 25-gauge hypodermic needle. Individuals raised in large broods had a lower body mass during adulthood than individuals raised in small broods (P=0.05; linear mixed model; see below for details), while individuals subjected to the high foraging cost treatment had a lower body mass than individuals subjected to the low cost treatment (P=0.01). These results validate our brood size and foraging cost treatment.

HAEMOLYSIS-HAEMAGGLUTINATION ASSAY

Blood samples were immediately transferred to a microfuge tube and kept cool before being centrifuged (5 min at 10,000 *g*). The plasma was separated from the cells and frozen at -20°C.

Levels of circulating NAbs and complement were estimated by means of the haemolysis–haemagglutination assay developed by Matson et al. (2005) within 10 days after blood collection. This assay is based on red blood cell agglutination and NAb-mediated complement activation. Quantification is done by a serial dilution (1:2) of plasma samples, starting with 50 µl plasma, with phosphate-buffered saline in a 96-well assay plate, which was then incubated with the same amount of rabbit red blood cell suspension for 90 min at 37ºC. Titers were scored blindly with respect to experimental brood size, foraging cost treatment, sex and age as the highest dilution step at which agglutination (NAb) and lysis (complement) of red blood cells was observed. Higher scores were associated with stronger immune responses. Half scores were assigned when the termination of the reaction was intermediate between two wells.

STATISTICAL ANALYSIS

Agglutination scores ranged between 3 and 8 (mean ± SD = 4.65 ± 0.73) and were treated as samples from a normal distribution. Lysis scores ranged between 0 and 2 (mean ± SD = 0.17 ± 0.36) and were treated as samples from a Poisson distribution to allow for the non-negative values for lysis scores despite the small mean. While lysis scores cannot be treated as count data *sensu stricto*, noninteger response variables can be included when modelling Poisson-distributed data in SAS 9.2 (SAS Institute, 2002–2008; see also Dugdale et al. 2010). Linear mixed models with restricted maximum likelihood and Kenward-Roger approximation for the degrees of freedom (Kenward and Roger 1997) were used to model agglutination scores. Generalized linear mixed models with logarithmic link function were fitted to model lysis scores. Starting models contained fixed factors BROOD SIZE (small or large), FORAGING COSTS (high or low) and SEX, all two-factor interactions and the three-factor interaction. Models were corrected for the covariates AGE, BODY MASS, TREATMENT DURATION

and ORIGINAL BROOD SIZE (i.e. in the nest of origin) to account for differences in age, body mass, time involved in the foraging cost treatment and brood size shift, respectively. BODY CONDITION INDEX (BCI; calculated as the residual of the linear regression of body mass on tarsus length with both measures being logarithmic transformed) was not included in the models to avoid multi-collinearity problems due to its high correlation with body mass (ρ=0.93; P<0.0001). NEST OF REARING and AVIARY were included as random factors in all models to avoid pseudoreplication from sharing a common nest and adult environment. Non-significant fixed effects were removed from the model following a backward stepwise procedure. All analyses were performed with procedures Mixed (agglutination) and Glimmix (lysis) in SAS 9.2.

Results

Overall, lysis scores did not differ between foraging cost treatments, brood sizes or sexes (all $P>0.19$). However, lysis scores significantly differed between brood sizes and sexes when accounting for the foraging cost treatment that adults were exposed to (interactions between brood size and foraging costs $P=0.021$, and between sex and foraging costs $P=0.022$; Table 1). Under high foraging costs, lysis scores did not differ between individuals raised in large and small broods (*post-hoc* test: $t_{54}=-1.21$; $P=0.23$; Fig. 1A) or between males and females (*post-hoc* test: $t_{54}=0.96$; $P=0.34$; Fig. 1B). In contrast, among low-cost foragers, lysis scores were significantly smaller in individuals raised in large broods (*post-hoc* test: $t_{54}=2.12$; $P=0.038$; Fig. 1A), and in females (*post-hoc* test: $t_{54}=-2.29$; $P=0.026$; Fig. 1B).

TABLE 1 AND FIGURE 1 ABOUT HERE

In accordance with lysis scores, average agglutination scores did not vary with the brood size manipulation and foraging cost treatment, nor did they differ between the sexes (all $P>0.19$). However, in contrast to the former, the effect of the foraging treatment did not depend on whether individuals had been raised in small or large broods ($P=0.71$). Likewise, there was no effect of either treatment (or their interaction) when the sex of the individuals was taken into account (all $P>0.57$).

Lysis and agglutination scores were not related with age or body mass, neither did they vary with the time that an individual was involved in the foraging cost treatment or the original brood size (all $P>0.22$).

Discussion

By experimentally manipulating environmental conditions in nestlings (brood size experiment) and adults (foraging cost treatment), we showed that lysis titres, but not agglutination titres, were affected by nestling environmental conditions and sex, depending on the adult environment. Lysis scores of low-cost foragers (i.e. favourable adult condition) were significantly lower if these individuals had been raised in large broods, whereas scores of high-cost foragers (i.e. unfavourable adult condition) were independent of brood size. Long-term negative effects of development under unfavourable conditions may either be expected (i) to be evident under both favourable and unfavourable adult conditions; (ii) to be evident under unfavourable adult conditions only, or (iii) to be stronger under favourable adult conditions because of the mismatch between nestling and adult conditions (see introduction). Though higher lysis scores under matching nestling and adult conditions (Fig. 1A) seem to provide support for the latter prediction, the non-significant effect of brood size under high foraging costs rather implies another mechanism, i.e. unfavourable nestling conditions causing long-term negative effects on (at least one aspect of) an individual's physiology but being evident under favourable adult conditions only. It has earlier been shown that increased foraging costs result in physiological adjustments and decreased investment in somatic maintenance to save energy (Wiersma et al. 2005; Wiersma and Verhulst 2005). Therefore, birds experiencing high foraging costs might simply have withdrawn energy from the maintenance of innate immunity, because of the associated energetic costs (Klasing 2004). If so, long-term effects of early developmental conditions might be masked because of the reallocation of the energy budget.

Alternatively, it is also possible that effects under unfavourable conditions were masked due to differential mortality. High-cost foragers (Wiersma et al. 2005; Wiersma and Verhulst 2005) and individuals raised in large broods (e.g. Alonso-Alvarez et al. 2006; Tschirren et al. 2009; Verhulst et al. 2006) usually experience negative effects on a variety of fitness-related traits, in particular under unfavourable adult conditions (e.g. Hendrickx et al. 2003; Lens et al. 2002; Talloen et al. 2010). Indeed, high-cost foragers suffered from higher mortality when raised in large broods, whereas a similar effect was not apparent in low-cost foragers. If differential mortality under a combined 'large brood/high foraging cost' treatment was biased towards individuals with a low immune response (Møller and Saino 2004), the average immune response of surviving (and thus blood-sampled) adults would be expected to increase, and long-term effects on immunity of high-cost foragers to be masked. Unfortunately, we did not measure immune response when birds fledged in order to test this hypothesis. The fact that lysis scores in low-cost foragers (no bias in mortality) were smaller when raised in large broods supports a long-term cost of brood size on innate immunity. Our results contrast those of a study on free-living great tits (*Parus major*) where (morphological) differences between individuals that developed in favourable and adverse conditions were only apparent under suboptimal conditions (Talloen et al. 2010). Yet, we propose that the same mechanism may be acting in both studies (i.e. adverse consequences of developmental stress being more severe in unfavourable adult conditions), but causing different effects in adulthood depending on whether or not differential mortality was involved.

Likewise, differential mortality between sexes may explain why female low-cost foragers had lower immune responses than males, while this sexual dimorphism in immune function was not evident when foraging entailed higher energetic costs. Given the higher mortality rates in female zebra finches under harsh environmental conditions (Bradbury and Blakey 1998; de

Kogel 1997b; Kilner 1998), we suggest that mortality rates were biased towards females with relatively weak immune capacity such that the remaining females mounted higher immune responses, on average (see also Møller and Saino 2004). Alternatively, seeing that immunity is a highly plastic trait that varies sex-specifically and in a context-dependent way (Love et al. 2008), resource allocation trade-offs between immunity and other functions may differ between sexes. Male high-cost foragers may prioritize the investment in structures associated with mate acquisition, such as plumage ornaments, at the expense of immunocompetence to have similar mating success than individuals living in favourable conditions (Arnold et al. 2007; Naguib and Nemitz 2007). In contrast, females may rather maximize their survival through investments in immunity (Møller and Saino 2004). As a result, the immune responses of both sexes might be similar under poor environmental conditions.

Inverse effects of brood size on lysis titres of low-cost foragers are in agreement with the negative relationships of brood size with PHA response (Alonso-Alvarez et al. 2006; Naguib et al. 2004) earlier observed in zebra finch nestlings (but not in adults, see Tschirren et al. 2009). Several hypotheses have been invoked to explain these patterns. First, immune responses in large broods might be reduced as a result of the restricted amount of resources that nestlings receive from either parent (e.g. Fargallo et al. 2002; Hõrak et al. 1999; Saino et al. 1997). Second, lower immune responses in large broods might be caused by increased sibling competition resulting in an increase in testosterone (Naguib et al. 2004) and a suppressed immune function (Boonekamp et al. 2008; Fargallo et al. 2007; Folstad and Karter 1992; but see Roberts et al. 2004). Evidence for immunosuppressive effects of testosterone, however, is still lacking for zebra finches. Third, it has been suggested that stress caused by higher sibling competition increases corticosterone levels which, in turn, may suppress immunity (Saino et al. 2003). Correspondingly, prolonged fasting resulted in increased

corticosterone and decreased NAb levels in female mallards (Bourgeon et al. 2010). Yet, administering corticosterone did not alter innate immune capacity in barn owl (*Tyto alba*) nestlings (Stier et al. 2009), while brood size manipulation produced no effect on corticosterone in nestlings of the spotless starling (Sturnus unicolor; Gil et al. 2008), and have not been measured in zebra finches. However, as we only measured two components of the innate immune system, we cannot rule out that other branches of (innate) immunity were differently affected. It is possible that individuals optimized their investment in each component of the immune system, depending on the costs and benefits of investment in a particular component and on the conditions they encounter (Norris and Evans 2000).

Yet, results of this study do not allow to fully exclude an alternative mechanism, i.e. that individuals raised in large broods possessed a lower ability to obtain food during adulthood. If so, the lower innate immune responses in individuals raised in large broods would not be caused by a long-term effect of nutritional conditions during early development but rather by a reduced resource consumption during adulthood and, as a result, a lower investment in the maintenance of the immune system. Yet, we think that such mechanism is unlikely to operate in our study population, as the time spent eating and dominance over food during adulthood are not affected by brood size in zebra finches (de Kogel 1997a) and body mass and lysis score were not correlated in this study. Furthermore, without knowledge of an individual's degree of pathogen infection, high immune responses can be interpreted in at least two different ways, i.e. as an indication of a well-functioning immune system (indicative of good individual condition), or as a consequence of intense pathogen infection (indicative of poor condition; Norris and Evans 2000). In the latter case, high lysis scores in birds from small broods would indicate that these birds are exposed to a higher number of infections. Yet, while NAb levels have been shown to rise in response to an experimentally increased level of

parasite exposure, a similar effect was not found for complement activation (De Coster et al. 2010).

Unlike lysis scores, agglutination scores were not affected by brood size or foraging cost treatments, nor associated with a bird's sex. Likewise, NAb concentrations did not vary with clutch size or food limitation in several bird species (e.g. Buehler et al. 2009a; Lee et al. 2008), but significant relationships have been found with growth rate, mass differences among nestlings, prolonged fasting, body mass and environmental conditions in other species (e.g. Bourgeon et al. 2010; Buehler et al. 2009b; Mauck et al. 2005; Parejo et al. 2007). The fact that agglutination titres did not vary with the experimental treatments in our study suggests that NAbs are not (or only slightly) affected by environmental conditions in zebra finches or that the species actively buffers NAbs against stress-induced changes (see also Buehler et al. 2009a), possibly at the expense of investment in other life-history traits. Further experimental study is needed to discriminate between both hypotheses.

To summarize, long-term effects of developmental conditions on physiology were only apparent under favourable conditions in adulthood. We showed that one component of innate immune function of adult zebra finches is affected by their condition during development, and varies between sexes. To better understand the putative adaptive mechanisms underlying these relationships, future experiments that take into account resource-allocation trade-offs among multiple condition-dependent traits are required.

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Figure legends

Fig. 1 Effects of foraging cost manipulations on lysis scores in relation to (A) experimental brood size and (B) sex. Small (S) and large (L) broods and female (F) and male (M) birds are represented by filled and open bars, respectively. Numbers represent samples sizes; error bars represent standard errors.

Tables

Table 1 Generalized linear mixed model analysis of lysis scores in relation to brood size manipulation, foraging cost treatment and sex. Significant terms are indicated in bold.

Model term	DF	F	*P*
sex	1, 54	1.18	0.28
brood size	1, 54	0.50	0.48
foraging costs	1, 54	0.12	0.73
sex × brood size	1, 53	0.02	0.88
sex × foraging costs	**1, 54**	**5.54**	**0.022**
brood size × foraging costs	**1, 54**	**5.64**	**0.021**
sex × brood size × foraging costs	1, 52	0.08	0.77